AD_____

AWARD NUMBER: W81XWH-11-1-0429

TITLE: Microvascular Channel Device to Study Aggressiveness in Prostate Cancer Metastasis

PRINCIPAL INVESTIGATOR: Sayeda Yasmin-Karim

RECIPIENT: University of Rochester
Rochester, NY 14627-0140

REPORT DATE: August 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2014	Annual Summary	15 May 2011--14 May 2014

4. TITLE AND SUBTITLE Microvascular Channel Device to Study Aggressiveness in Prostate Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0429

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sayeda Yasmin-Karim

E-Mail: Sayeda_yasmin-karim@urmc.rochester.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester

Rochester, NY 14627-0140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To study circulating prostate cancer cell adhesion behavior and the contribution to distant metastasis of prostate cancer, we applied a dynamic flow-based E-selectin+SDF-1 coated micro-channel model to isolate and characterize a subpopulation of adhering prostate cancer cells. We found a positive correlation of cells' aggressiveness with their adhesion/rolling ability. By using this device we then sorted prostate cancer cell lines (PC3 and DU-145) into rolling and floating populations, and tested for their behavior *in vitro* and *in vivo*. Results from our *in vitro* studies found that rolling cancer cells are more aggressive in nature with higher growth rate and invasion and form bigger and more colonies in soft agar and 3D sphere formation assays. In our *in vivo* study of orthotopic implantation mouse model, we found that sorted rolling cells are more potent in cases of tumor formation and distance metastasis. In summary, this flow based device provides a platform for isolating the most dangerous, aggressive form of cancer cells which can be used for isolating and characterizing human patients' peripheral blood and to form a process for customized cancer treatment.

15. SUBJECT TERMS

prostate cancer, metastasis, selectin, circulating, Micro-vascular

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
1. Introduction	2
2. Keywords	2
3. Overall Project Summary	2-16
4. Key Research Accomplishments	16
5. Conclusion	17
6. Publications, Abstracts, and Presentations	18
7. Inventions, Patents and Licenses	19
8. Reportable Outcomes	19
9. Other Achievements	19
10. References	20-21
11. Appendices	22
12. Training or Fellowship Award	22-23

INTRODUCTION

Prostate cancer (PCa) is the second most common cancer in men in America. Metastasis is the most dreaded stage of cancer, which accounts for the vast majority of prostate cancer related deaths. (Aguirre-Ghiso, 2007; Jacobs and Sackstein, 2011; Lange and Vessella, 1998). Identifying the population at risk for developing advanced PCa and preventing metastases is one of the greatest challenges in cancer treatment today. Cancer metastases are the culmination of a complex series of steps where cancer cells degrade extracellular matrices allowing them to break away from the primary tumors. Circulating tumor cells (CTCs) preferentially roll and adhere on bone marrow vascular endothelial cells where abundant E-selectin and stromal cell-derived factor 1 (SDF-1) are expressed, subsequently initiating a cascade of activation events that eventually lead to the development of metastases (Barthel et al., 2007; Barthel et al., 2013). To elucidate the importance of circulating prostate cancer cells' rolling and adhesion behaviors in the development of metastasis, we applied a dynamic cylindrical flow-based microchannel device that is coated with E-selectin and SDF-1, mimicking capillary endothelium, and allowing prostate cancer cells to roll and adhere under flow. In this report we elucidate the roles of circulating PCa cells' rolling and adhesion behavior in the development of aggressive metastatic PCa. We applied a dynamic flow-based E-selectin/SDF-1 coated microchannel system, mimicking post capillary venules, as we described previously (Hsu et al., 2011). We demonstrated that circulating PCa cells' rolling/adhesion capacity contributes to PCa's distant metastasis, which is mediated via an E-selectin ligand, ESL-1. Consequently, the interaction of E-selectin/ESL-1 transduces a cascade of signaling which facilitates prostate cancer metastasis.

KEYWORDS

Prostate cancer, circulating prostate cancer cell, rolling cancer cell, E-selectin, ESL-1.

OVERALL PROJECT SUMMARY

A. Major goals of the project:

Task 1: Correlation of cancers' aggressiveness with their adhesion /rolling capacity in static and dynamic flow-based status (timeframe: months 1-12).

1a: Determination of cell rolling capacity in a dynamic state (timeframe: months 1-12).

1b: Determination of cell adhesion ability in a static condition (timeframe: months 1-8).

1c: Determination of cell metastatic behavior *in vitro* (timeframe: months 1-12).

Task 2: Sorting prostate cancer cells into adhesion (attached) and non-adhesion (floating) subpopulations and comparison of these two populations' metastatic behaviors *in vitro* and *in vivo* (timeframe: months 5-24).

2a: Fractionation of prostate cancer (PRCA) cells based on rolling capacity (timeframe: months 5-20).

2b: Characterization of sorted cells metastatic behavior *in vitro* (timeframe: months 7-18).

2c: Confirmation of metastatic potential on those rolling PRCA cells *in vivo* by the orthotopic injection mice model (timeframe: months 13-24).

Task 3:

3a: Verify up-regulation of metastatic promoting genes in rolling PCa cells by the Scatter Plot analysis using the metastasis pathway SuperArray (timeframe: months 7-12).

3b: Using the genome wide gene array profiling to build the network that controls PCa cell adhesion and metastasis (timeframe: months 13-24).

3c: Determination of those metastatic related genes' impact to cell rolling capacity and metastatic behavior *in vitro* (timeframe: months 15-30).

3d: Determination of those metastatic related genes' impact to cell rolling capacity and metastatic behavior *in vitro and in vivo* (timeframe: months 25-36).

B. Accomplishment under these goals:

Task 1 Accomplishment:

We completed task 1and reported this in our first report.

Task 2 Accomplishment:

We completed tasks 2a and 2b and reported this in our first report.

We completed task 2c and reported this in our second report.

Task 3 Accomplishment: We completed task 3 and following is a brief report:

Determination of the aggressive/metastatic related gene

I. 1. ESL-1 expression is high in rolling cells and tissue

When PCa cells come in contact with E-selectin coated surfaces, some cells tether, roll, and ultimately adhere to the surface (Dimitroff et al., 2004). It has been shown by affinity or binding assay that E- selectin can recognize a diverse and structurally distinct glycoconjugates in various cancer cells which includes P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), a Glycoform of CD44, B2 integrin and glycolipids (Barthel et al., 2007). To discover the molecular characteristics that distinguish rolling from floating cells, we examined the expression levels of three common E-selectin ligands, CD44, ESL-1 or PSGL-1. We found that only ESL-1, but not CD44 or PSGL-1, showed a much higher induction in rolling PC-3 and DU145 cell lines than in floating cells (**Figure. I.1A**). More importantly, this elevated ESL-1 expression was also found in the prostate of mice with xenografted tumors from implanted rolling PC-3 cells, as compared to prostates of mice implanted with floating PC-3 cells by Western blotting analysis (**Figure I.1B**). ESL-1 was expressed in prostate tissues and their corresponding metastatic lung lesions by shown by immunohistogram (IHC) (**Figure I.1C**).

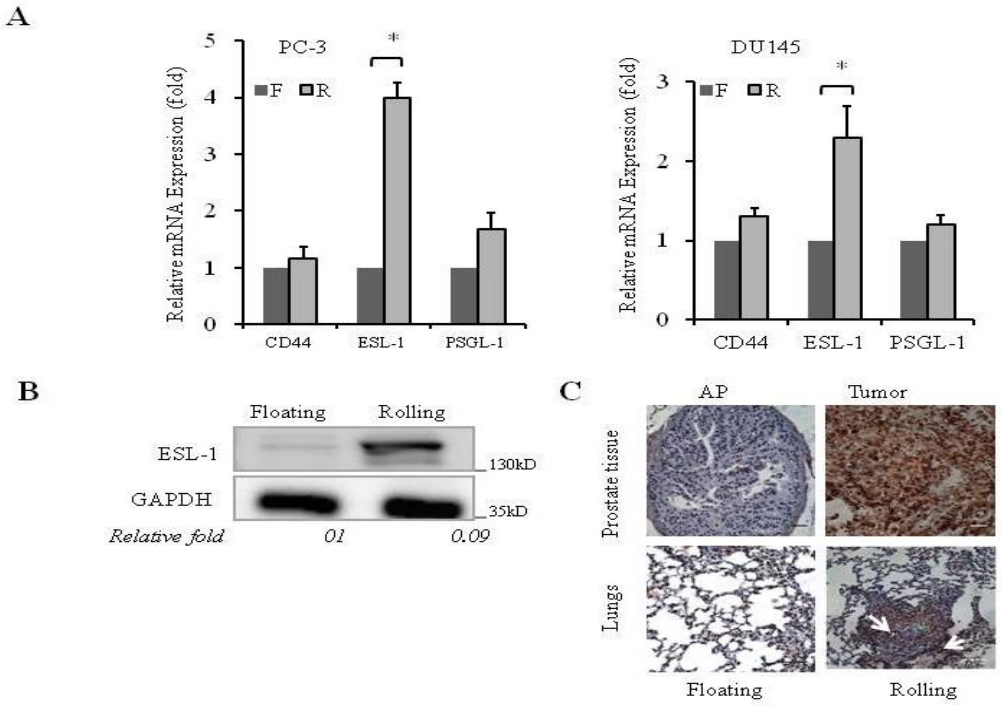


Figure I.1. ESL-1 was elevated in rolling PCa cells. (A) Relative mRNA expression levels of E-selectin ligands; CD44, ESL-1 and PSGL-1 in sorted PC-3 and DU145 cells were examined by QPCR. Bar graph represents the average of 3 experiments. Error bars indicate SEM. *, $P < 0.05$. F represents floating and R represents rolling and AP represent anterior prostate. **(B)** Western blot analysis of ESL-1 expression in the prostate tissues of xenografted mice implanted with rolling and floating PC-3 cells. **(C)** IHC with anti ESL-1 antibody of the same prostate tissues and their corresponding lungs.

I.2 Creation of shESL-1 and scESL-1 in PC-3 and DU145 PCa cells

Previous data demonstrate that there is higher expression of ESL-1, an E-selectin ligand, in rolling PCa cells as well as in mouse tissue. To determine further if ESL-1 is responsible for rolling PCa cells' aggressive phenotypes, we utilized RNA interference to knock down ESL-1 expression in PC-3 and DU145 cells. Two shESL-1 (shESL-1#4 and shESL-1#5) and one scramble (scESL-1) stable cell lines were generated in PC-3 and DU145 and the expression of ESL-1 was determined by QPCR and Western blot. As shown in **Figure I.2A and I.2B**, we successfully knocked down ESL-1 expression from PC-3 and DU145 cells. Both shESL-1#4 and shESL-1#5 ESL-1 clones express significantly lower amounts of ESL-1 than scESL-1.

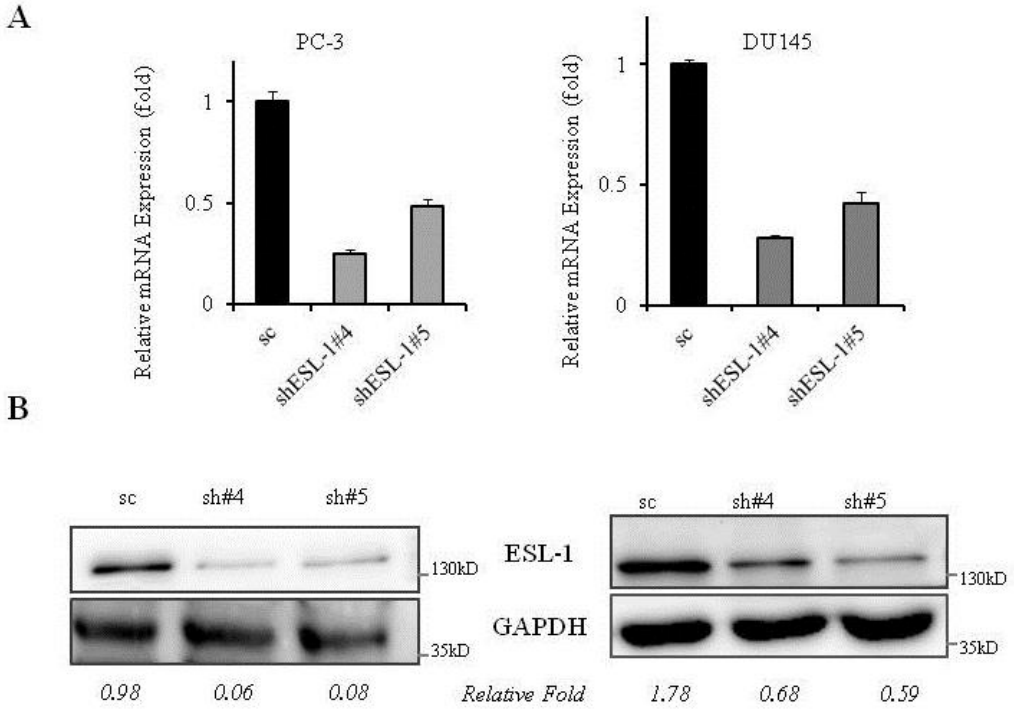


Figure I.2 Creation of shESL-1 and scESL-1 in PC-3 and DU145 PCa cells. (A) Expression of ESL-1 was determined by Q-PCR after knock down of ESL-1 expression by RNA interference. Bar graph represents the fold reduction of ESL-1 expression in shESL-1#4 and shESL-1#5 ESL-1 clones compared to scramble control in both PC-3 and DU145 cells. **(B)** Western blot analysis of ESL-1 expression in shESL-1#4 and shESL-1#5 ESL-1 clones compared to scramble control in both PC-3 and DU145 cells.

I.3. ESL-1 is responsible for PCa cells' rolling/adhesion

Both shESL-1 and scESL-1clones were subjected into different *in vitro* assays to test their aggressiveness phenotypes. First, stable shESL-1 clones and scramble control cells were perfused through the E-selectin+SDF-1β coated microtubes and their rolling capacity was analyzed. As expected, knocking down ESL-1 impairs PCa cells' rolling capacity significantly, where we found a significantly fewer number of shESL-1 PC-3 and shESL-1 DU145 cells can roll and adhere to the coated microtubes (**Figure I.3A**). We also examined their ability to adhere to HUVEC cells and found that shESL-1 PCa cells displayed significantly less static adhesion ability than scramble control cells (**Figure I.3B**).

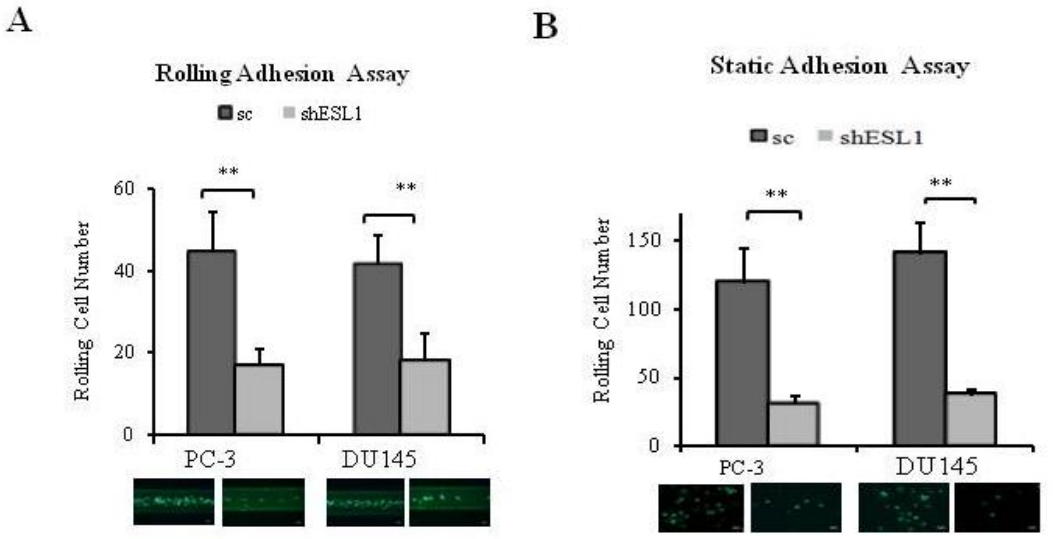


Figure I.3. ESL-1 is responsible for PCa rolling on E-selectin surface. (A) Examining the rolling cell numbers between the shESL-1and scESL-1 cells using the microchannel system under flow. Bar graph represents the average rolling/adhesion cell number of consecutive 10 frames (50X) of videos of one experiment. A representative picture was shown. Bar scale = 100μm. **(B)** Comparing the static adhesion behavior between the shESL-1and scESL-1 cells on HUVEC cells. Bar graphs represent the average adherent cell numbers per well and the picture represents 50x view of the shESL-1and scESL-1 of PC-3 and DU145 adherent cells. Bar scale = 50μm. Results represent the average of at least 3 experiments. Scale bar represents 100 um. Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

I.4. ESL-1 is responsible for PCa cells' aggressiveness

In addition to cell adhesion, we also tested if ESL-1 is important for cells to grow in a 3D anchorage-independent manner. We found that scESL-1control PC-3 cells grew many colonies, while strikingly those shESL-1of PC-3 cells did not develop colonies even after 28 days of culture (**Figure I.4A**). This result remains true in sphere forming assays for stem cells' property where scESL-1 of DU145 cells had a higher number and bigger size of spheres compared to shESL-1 cells (**Figure I.4B**). These data support a critical role of ESL-1 not only in adherence ability to the endothelial cells, but also in tumorigenesis, both of which are required for cancer metastasis.

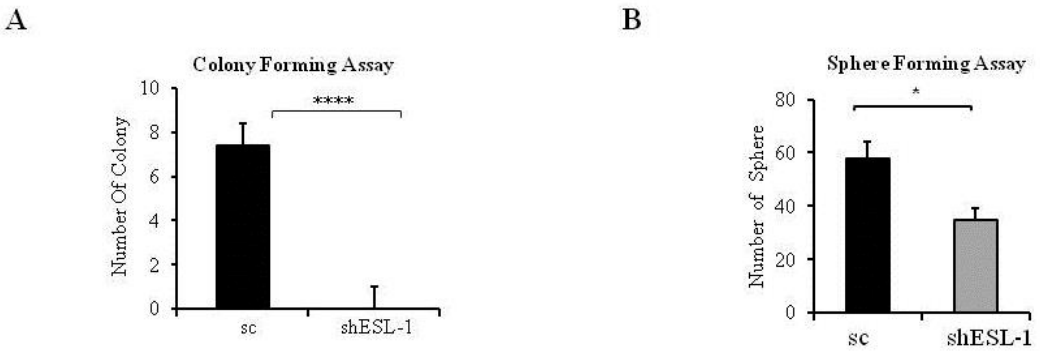


Figure I.4. ESL-1 is responsible for PCa aggressiveness. (A) Anchorage-independent soft agar colony formation assay with scESL-1and shESL-1 of PC-3 cell line. Colonies were counted after 28 days of culture. Bar graph represents the colony numbers per well in 6 well plates (each were triplicated). **(B)** 3D sphere forming assay to define the sphere forming ability as determinant of stemness of scESL-1and shESL-1 of DU145 cell line. Bar graph represents average sphere numbers in each well of 96 well plate. Results represent the average of at least 3 experiments. Scale bar represents 100 um. Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

I.5. ESL-1 expression correlates with clinical PCa progression and metastasis

We have identified ESL-1 as the key factor that controls circulating PCa rolling/adhesion, suggesting the expression levels of ESL-1 in circulating PCa might serve as a biomarker to predict PCa patients' outcomes. To assess the clinical relevance of ESL-1 in human PCa samples, the gene expression profile of ESL-1 was analyzed through public microarray repositories from NCBI Gene Expression Omnibus (Profile # GDS2546; Metastatic prostate cancer HG-U95B). We compared the expression levels of ESL-1 in three groups: (1) normal prostate tissue free of any pathological alteration (n=18), (2) primary PCa tumors (n=63) and (3) distant metastasis samples (n=25). We found high levels of ESL-1in metastatic tumors in comparison to normal prostate and primary tumors (**Figure I.5**), and no significant difference in normal prostate and primary PCa tumors. This elevated level of ESL-1 found in human PCa metastatic samples further supports ESL-1's roles in PCa metastasis; in particular, its status in circulating PCa cells could be an important functional biomarker for prediction of disease behavior.

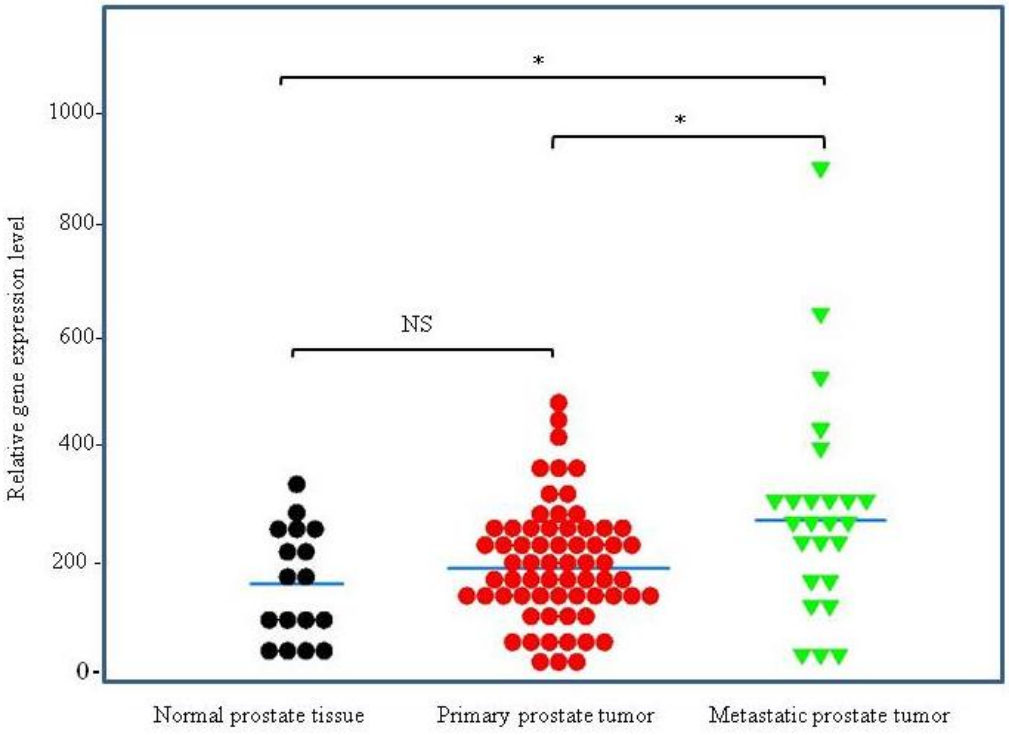


Figure I. 5. Elevated ESL-1 expression found in metastatic prostate cancer patients' tissues. To access the clinical relevance of ESL-1 in human PCa, the gene expression profile of ESL-1 was analyzed through public microarray repositories from NCBI Gene Expression Omnibus (Profile # GDS2546; Metastatic prostate cancer HG-U95B). Box plots represent expression level of ESL-1 in following 3 categories: normal prostate tissue free of any pathological alteration (n=18), primary PCa tumors (n=63) and distant metastasis samples (n=25). *, $P < 0.05$. NS represents non specific.

II. Identifying the possible ESL-1 modulated signaling pathway.

II 1. ESL-1 may activates RAS-MAP kinase signaling pathway

The activation of MAP kinase pathways has been associated with cell migration and invasion, as well as more aggressive behaviors (Guo et al., 2013). Of particular importance, activation of MAP kinase is associated with cancer metastasis, and elevated levels of phosphorylation of key signals such as ERK1/2 are often found in some cancer invasion pathways. Therefore, we tested if ESL-1, in contact with E-selectin, could activate MAP kinase signal in PCa cells. We found that knockdown of ESL-1 significantly reduced nRAS, phosphoMEK, phosphoERK1/2 expression and its downstream transcription factor, cFos as compared to scramble ESL-1 cells (**Figure II.1 right panel**). Importantly, the elevated levels of MAP kinase signaling, such as nRAS, pMEK, pERK1/2 and cFOS, are also found in the prostate

tumors derived from the xenografted mice who received high ESL-1 rolling PC-3 cells (**Figure II.1 left panel**) and DU145 cells (**Figure II.1 middle panel**).

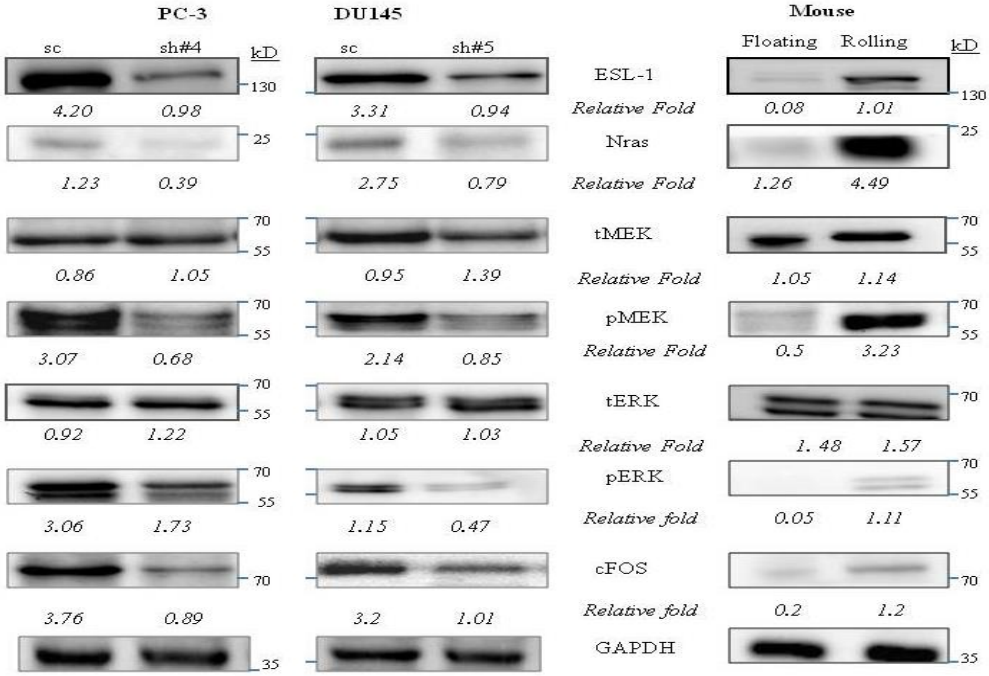


Figure II.1. ESL-1 activates RAS-MAP kinase signaling pathway. To study the underlying mechanism by which ESL-1 controls Western blot analysis of shESL-1 and scESL-1 of PC-3 and DU145 cells and orthotopic rolling and floating prostate tissues were compared using ESL-1, Nras, total MEK, phosphoMEK, total ERK1/2, phosphoERK1/2, and its downstream transcription factor, cFOS antibody. GAPDH was used as a housekeeping gene.

II.2. Actin, Myosin and Rho related may controlled by ESL-1

These data implied that PCa cells express ESL-1 that interacts with E-selectin on the surface of endothelial cells, and this interaction would then induce a cascade of signaling in PCa cells, consequently altering their behavior to invade and grow in a second target organ. The downstream targets of ESL-1 in controlling cancer metastasis were investigated by applying the integrin-mediated cell adhesion and migration PCR array (Bio-Rad), because selectins and their ligands orchestrate the cell mobility/adhesion mainly through an integrin dependent mechanism. As shown in **Figure II.2**, we found that a number of actin and myosin related genes including Rho family were down-regulated in the shESL-1 cells as compared to scramble control cells. Our data indicate, for the first time, that ESL-1 is the key factor in circulating PCa cells' rolling

behavior. The activation of ESL-1 would then transduce MAP kinase and its associated downstream target genes, such as cytoskeleton related proteins, consequently altering the cell mobility, and, adhesion, and eventually facilitating cancer metastasis.

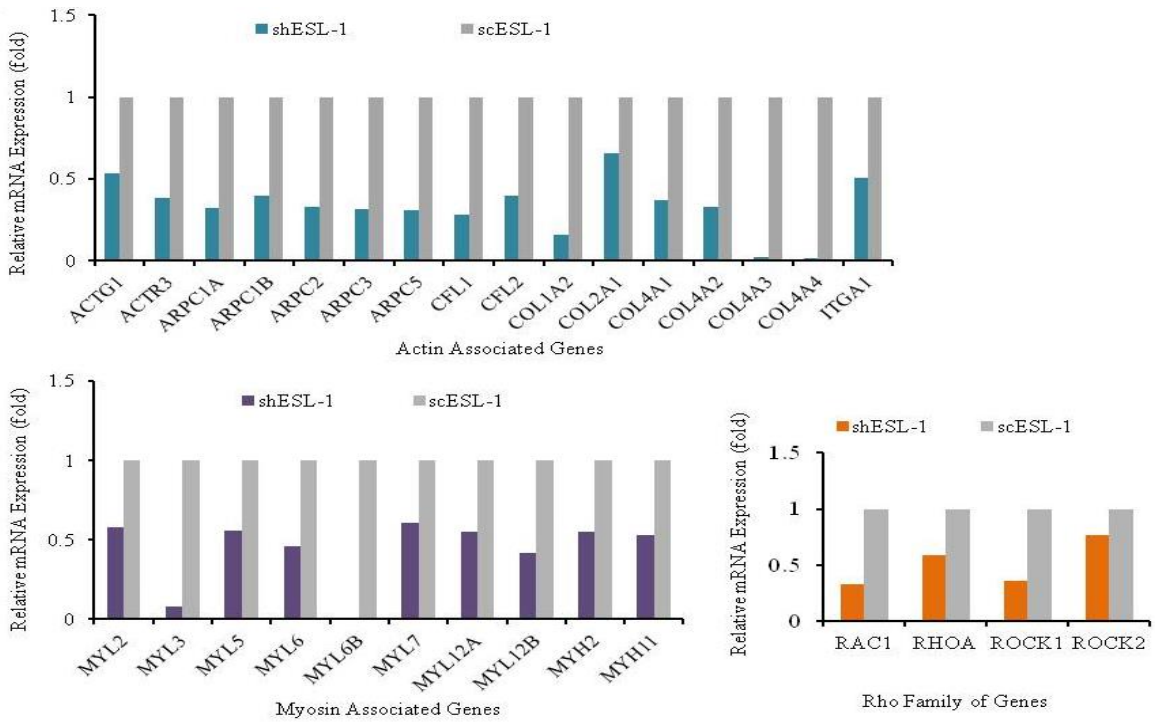


Figure II.2. Identifying ESL-1 downstream targets in the integrin-mediated cell adhesion pathway.
Integrin-mediated cell adhesion and migration PCR array was performed to compare the cytoskeleton gene expression in scESL-1 and shESL-1 PC-3 cell line. Bar graph represents the relative fold expression 3 clusters of genes representing Actin, Myosin, and Rho family.

As summarized in **Figure III**, circulating PCa cells expressing high level of ESL-1 can be stalled and roll on the endothelial cells where E-selectin is expressed. Through the interaction with integrin and bone specific SDF-1 in the endothelial cells, rolling PCa cells then form firm adhesion, transmigration, and eventually complete the extravasation of cancer cells to the metastatic site. Our studies further demonstrated that the interaction of E-selectin with ESL-1 in PCa cells triggers a signaling kinase cascade, from activation of RAS, MEK, and ERK, consequently inducing the expression transcription factors; and activates a cascade of gene expressions that allows cancer cells to migrate, invade and spread to the secondary organs.

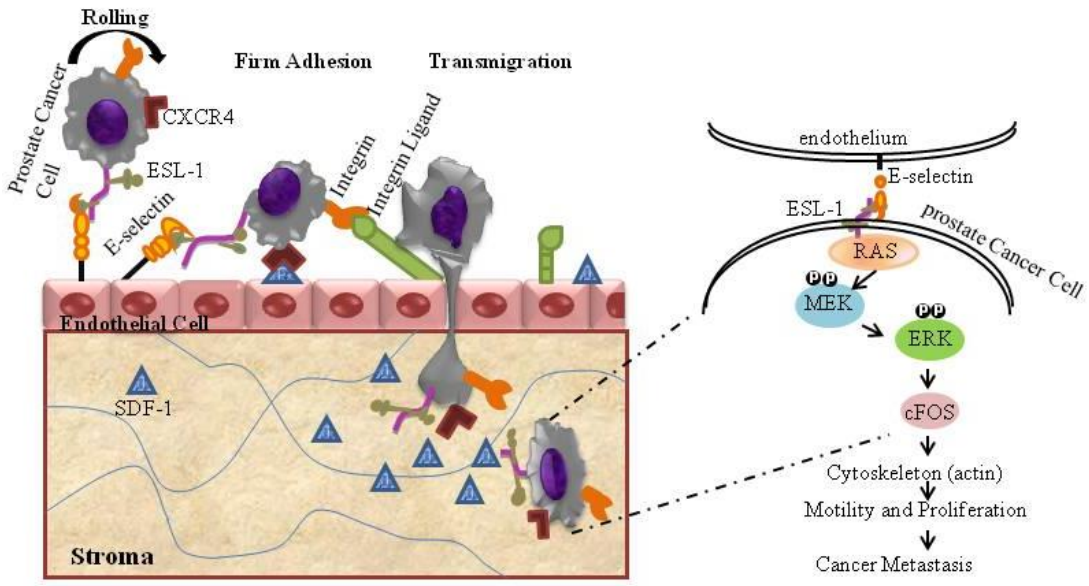


Figure III. Schematic diagram of the oncogenic signaling pathways induced by E-selectin/ESL-1- mediated PCa rolling and adhesion on endothelial cell surface. PCa cells express high level of E-selectin ligand, ESL-1, which mediates the rolling,/adhesion of PCa cells on endothelial surface through the RAS-ERK- MAPkinase pathway.

Materials and Methods

mRNA extraction and conversion to cDNA. Cells were lysated using TRIZOL reagent (Invitrogen) and RNA was extracted following the recommended protocol and converted to cDNA by C1000 Thermal Cycler PCR machine (Bio-Rad) by reverse transcriptase polymerase chain reaction (RT-PCR) following manufacturer's (Invitrogen) protocol.

Semiquantitative RT-PCR and Real-time PCR Assay. Total RNA was extracted from cells using Trizol (Invitrogen) following the company protocol, extracted RNA was measured by Nanodrop 2000c spectrophotometer and converted to cDNA by RT-PCR with reverse transcription by the iScript reversetranscriptase kit (Bio-Rad) using C1000 Thermal Cycler (Bio-Rad) following the protocol: 25 for 300s, 42 for 1800s, 85 for 300s 4 for 10s n. Real-time PCR was done with SYBR Green PCR Master Mix on an C1000 Thermal Cycler CFX96 multicolor real-time PCR detection system (Bio-Rad) following protocol: 50^0C for 2 min, 95^0C for 10 min then 40 cycles of denaturation at 95^0C for 15 sec, annealing at 60^0C for 1 min and extension at 55^0C for 10sec and 95^0C for 5 sec.

Western Blot Analysis. Whole cell/tissue lysates were prepared by lysating cells in Radioimmunoprecipitation assay (RIPA) buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) with protease inhibitor cocktail tablet (complete ultra tablet mini, Roche) on ice. Lysates were centrifuged at 14000rpm for 20 minutes at 4-8^{0}C. Supernatants were collected and protein concentration was measured by Nanodrop 2000c spectrophotometer using Bio-Rad protein assay dye reagent (Thermo Fisher). 10 μg protein for each sample was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 2 hours at room temperature with 5% BioRad blotting grade blocker nonfat dry milk dissolved in TBST [10 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 0.05% Tween 20]. The membrane was then incubated with primary antibodies diluted in TBST+5% BioRad blotting grade blocker nonfat dry milk at 4°C overnight following the company recommended dilution. The membrane was then washed with TBST and then incubated with Horse Radish Peroxidase (HRP)–conjugated secondary antibodies (Cell Signaling) for 45 minutes at room temperature. The membranes were washed in TBST (3 times for 10 minutes each at room temperature) and the immunoreactive bands were visualized by alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium phosphatase substrate (Thermo Scientific) using Bio-Rad ChemiDoc XRS+ imaging system.

Antibodies. Antibodies were used according to manufacturer recommended dilution (from 1:100 to 1:5000) and HRP-conjugated secondary antibody was used to 1:2000 to 1:10,000 dilution.

Primary Antibodies:

1. ESL-1: cell Signalling (Cat # ab103439, rabit mAb)
2. Nras (F155): Santa Cruz (Cat# Sc-31, mouse mAb)
3. Total MEK: Cell Signalling (Cat# 4694s, Rabit mAb)
4. Phospho MEK : Cell Signalling (Cat# 2338s, Rabit mAb)
5. Total ERK1/2: Cell Signalling (Cat# 4695, Rabit mAb)
6. Phospho ERK1/2: Cell Signalling (Cat# 4376, Rabit mAb)
7. cFOS: Senta Cruz (Cat# sc-52, mouse mAb)
8. GAPDH: Senta Cruz (Cat# sc-32233, mouse mAb)
9. βTubulin: Cell Signalling (cat# 03/2013, rabit mAb)
10. Nanog: Abcam (ab80897, Rabbit mAb)

Secondary Antibodies:

1. Goat anti-mouse IgG-HRP: sc-2005 (SentaCruz)
2. Anti-Rabbit igG-HRP: #7074 (Cell Signalling)
3. Donkey anti-goat IgG-HRP: sc 2010 (Senta Cruz)

Immunohistochemistry (IHC) staining. IHC staining was performed following the protocol. The method of staining and microscopy was as follows: the formalin-fixed and paraffin-embedded sections (5 μm) of samples were dewaxed, and then rinsed by phosphate buffered saline. The sections were stained with the primary antibody (ESL-1) before being analyzed with the commercial kit. Negative control experiments were performed, in which phosphate buffered saline was used instead of the primary antibody. Finally, the sections were counterstained with hematoxylin (Ricca Chemical Company Catalogue No. 3530-16) and rinsed by tap water. Sections were observed and the micrographs were pictured using Leica DM5000 B microscope coupled with Optronics MacroFire digital camera.

Establishment of shESL-1 PCa Cells. ESL-1 shRNA and universal scramble control plasmids were constructed in lentiviral pGIPZ vector (Open Biosystems) following the company protocol. To establish the stable cells we transfected pGIPZ -scramble or ESL-1 shRNA (pGIPZ -ESL-1 shRNA) with pGIPZ - psPAX2 (virus-packaging plasmid), and pMD_2G (envelope plasmid) into 293T cells using Lipofectamine 2000 (Invitrogen). The viral containing supernatants were collected 48-72 hours after transfection, infected into prostate cancer cells (PC-3 and DU145) and selected for stable gene insertion under 2 μg/ml puromycin.

Sequence of shESL-1 #4 is sense AAACTTGTAAGAAAACCGA,

Sequence of shESL-1 #5 is sense AAACTTGTAAGAAAACCGA,

Sequence of scESL-1is sense ATCTCGCTTGGGCGAGAGTAAG and antisense CTTACTCTCGCCCAAGCGAGAG.

Cell adhesion and migration PCR array. The downstream targets of ESL-1 in controlling cancer metastasis were investigated by applying the integrin-mediated cell adhesion and migration PCR array (Bio-Rad).

Discussion

During cancer metastasis, CTCs follow a selectin based migratory pathway that is similar to leukocytes during the inflammatory response (Bonnomet et al., 2010; Gajula et al., 2013;

Julien et al., 2011; Ley et al., 2007; Sackstein, 2004). Dimitroff et al have shown that E-selectin ligands, such as PSGL-1, are over-expressed in human prostate bone metastasis tissues (Dimitroff et al., 2004), suggesting a critical role for E-selectin ligands in cancer metastasis. To date, few studies have reported on the correlation between expression of the E-selectin ligands and prostate cancer bone metastasis (Barthel et al., 2007; Barthel et al., 2008; Dimitroff et al., 2005; Gout et al., 2008). In this study, we identified ESL-1 as the key factor that controls PCa cells' rolling capacity under the flow, and knocking down ESL-1 impairs PCa cell rolling and mobility, and disrupts anchorage independent growth. This study is the first study that demonstrates the functional roles of ESL-1 in controlling PCa rolling/adhesion and metastasis. ESL-1 is a Ca^{2+} dependent inducible cell adhesion glycoprotein, responsible for converting the initial tethering into slow rolling in myloed cells (Hidalgo et al., 2007). It is also believed to be a variant of the receptor of fibroblast growth factor (FGF) (Mourelatos et al., 1995; Steegmaier et al., 1995), which may demonstrates the interesting relationship between the high expression of ESL-1 with PCa growth rate, as FGF is known to be one of the growth factors in cancer progression (Turner and Grose, 2010).

The ESL-l mediated pathways that control cancer cell adhesion/rolling and metastasis are largely unknown. In this report, we showed that overexpression of ESL-1 activates Ras-MAP kinase cascade in PCa cells, then triggers the expression of downstream targets, like integrin mediated cytoskeleton system, such as actin, myosin polymerization, and the Rho family of proteins (Parsons et al., 2010). The ERK signaling pathway is known to control diverse cellular processes, and was found to be often up-regulated in human tumors. ERK signal has been found to be important for the osteomimetic properties of the bone metastasis of prostate cancer cell lines (Zayzafoon et al., 2004), and expression of the key signal factors such as MEK5 is found to be overexpressed in PCa metastatic tissues (Mehta et al., 2003) and can promote PCa cell mobility. Moreover, we also identified ESL-1 downstream targeted genes that are involved in the cytoskeleton movement. All of these proteins are required for cell mobility, cell migration, and cell adhesion and all those cellular behaviors are essential for cancer progression during distant metastasis. In summary, our study reported a novel pathway that controls cell rolling and adhesion, from ESL-1 to RAS-ERK, then to cytoskeleton proteins. Targeting ESL-1 and/or its regulatory signal molecules, and/or downstream oncogenic proteins may provide a novel therapeutic strategy, especially for those patients who have a high ESL-1 expression in CTCs.

Our findings on ESL-1's oncogenic properties and its elevated expression proile in clinical metastases are clinically significant. First, ESL-1 can be used as a novel functional biomarker for CTCs that can be used to predict patients' outcomes, i.e., to include not only patients' CTCs numbers, but also the expression level of ESL-1 to determine CTCs' "bioactivity". More sensitive assays, such as an immunofluorescence assay, and an in-depth large scale clinical study are required to further establish the correlation of ESL-1 in CTCs with patients' outcomes. Second, this identified oncogenic pathway(s) induced by ESL-1 can serve as a principle for developing targeted therapy for those patients who have higher levels of ESL-1 in their CTCs.

Metastasis is the most dreaded stage of cancer and a major cause of death from prostate cancer. Therefore, understanding the PCa metastatic process, and identifying the essential molecular events during metastases are important tasks. Our study provides a model system to isolate and characterize a small rolling population of CTCs, and identifies a key molecule, ESL-1, for its roles in CTCs rolling and cancer metastasis. Further clinical evaluation of the expression levels of ESL-1 in patients and corresponding clinical outcomes are highly anticipated, and better therapeutics designed to block CTCs rolling and adhesion require further investigation.

KEY RESEARCH ACCOMPLISHMENTS

- Using this E-selectin/SDF-1 coated flow based device we can selectively collect the most aggressive prostate cancer cell population which also possesses prostate cancer stem cell like criteria.
- More aggressive prostate cancer cells possess higher rolling capacity on bone marrow endothelial surface.
- More aggressive prostate cancer cells express higher level of E-selectin ligand, ESL-1.
- Overexpression of ESL-1 may activate RAS-MAP kinase signaling pathway which enhance cytoskeleton movement to mediate rolling/adhesion for prostate cancer cell migration and promote proliferation.

CONCLUSION

In this thesis we have shown that E-selectin ligand, ESL-1, is the key molecule responsible for circulating PCa cells' rolling and adhesion. Importantly, knockdown of ESL-1 in PCa cells results in reduction of their rolling and adhesion ability consequently reducing the cancer aggressiveness. Moreover, upon examining the ESL-1 gene expression in GEO profile human database we found a positive correlation of ESL-1 expression with PCa stages. Molecular mechanisms revealed that ESL-1 can activate Ras-ERK-Kinase pathways and induce their downstream targets to influence cell movement and migration. This work is critical as we demonstrate that circulating PCa cells' rolling capacity contributes to cancer metastasis, which is in part controlled by ESL-1mediated signals. This ESL-1mediated oncogenic pathway represents a new therapeutic target.

According to the American Cancer Society, 2014: prostate cancer is the second leading cause of death in men in the United States of America. 1 in every 7 men will be diagnosed with prostate cancer during their lifetime and 1 in 36 will die from it. Metastasis related complication is the most common cause of death in prostate cancer patients (American Cancer Society, 2014). For early diagnosis, the most common currently used screening method is serum PSA level. But PSA cannot detect aggressiveness. So, we are lacking a better diagnostic system for aggressive/metastatic type prostate cancers and a method which can target the most aggressive/metastatic prostate cancer cells. The findings of this research will help to fill this need, but further studies need to be done. This study provides evidence for the critical roles of cancer cell rolling and its associated molecular events in cancer metastasis. This flow-based device might provide a platform for functionally characterizing CTCs' from human patients' samples and also personalize the treatment model of prostate cancer. ESL-1 can be used as a prostate cancer aggressiveness biomarker. Further, ESL-1 and its downstream pathway can be targeted to control prostate cancer metastasis.

PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS:

1. **Poster Presentation**: American Urological Association (AUA), Annual Scientific Meeting, Orlando, Florida, May 16 – 21, 2014.
   "E-selectin Ligand-1 Controls Circulating Prostate Cancer Cell Rolling/Adhesion and Metastasis".
   Authors: **Sayeda Yasmin-Karim**, Michael R. King, Edward Messing and Yi-Fen Lee.

Abstract: Circulating prostate cancer (PCa) cells preferentially roll and adhere to bone marrow vascular endothelial cells, where abundant E-selectin and stromal cell-derived factor 1 (SDF-1) are expressed, subsequently initiating a cascade of activation events that eventually lead to metastases. To elucidate the importance of PCa cells' rolling/adhesion behaviors for developing metastases, we developed a flow-based microchannel device that is coated with E-selectin and SDF-1, to allow PCa cells to roll/adhere. Using this devise, we captured a small fraction of rolling PCa cells with more aggressive cancer phenotype and stem cell-like characteristics, and developed metastatic tumors *in vivo*. Moreover, genes coding for E-selectin ligands, cancer stem cells and cancer metastases were found to be elevated in rolling PCa cells. Knock down of E-selectin ligand-1(ESL-1) on rolling cells significantly impaired PCa cells' rolling capacity and reduced cancer aggressive phenotypes. Moreover, ESL-1 activates RAS, consequently inducing MAP kinase cascade and its downstream targets to influence cell proliferation and migration. Our results indicated that PCa cells' rolling capacity contributes to PCa metastasis and this is in part controlled by ESL-1 and downstream signals. Thus, ESL-1 and its induced oncogenic pathways represent new therapeutic targets for controlling circulating PCa cells' rolling, adhesion and distant metastatic abilities.

2. **Submitted for publication:** "E-selectin Ligand-1 Controls Circulating Prostate Cancer Cell Rolling/Adhesion and Metastasis". **Sayeda Yasmin-Karim**, Michael R. King, Edward Messing and Yi-Fen Lee.

INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES:

Inventions:

- Technique to study the rolling behavior of circulating prostate cancer cells
- Identified a key molecule and its possible pathway responsible for prostate cancer cells' rolling and aggressiveness.

REPORTABLE OUTCOMES

We concluded that adhesion molecules play a major role in cancer metastatic process and this dynamic microchannel system allows us to capture the rolling CTCs and identify the specific gene profile for each individual cancer. Our results indicated that circulating PCa cells' rolling capacity contributes to PCa metastasis, and this is in part controlled by ESL-1 and may follow downstream MAP kinase signals. Thus, ESL-1 and its induced oncogenic pathways represent new therapeutic targets that control circulating PCa cells' rolling, adhesion and distant metastasis. The status of this molecule in circulating PCa cells could also be an important functional biomarker for prediction of disease behavior.

We also invented a technique to capture CTC's which will help in identifying the aggressive type of PCa cells to characterize and also to analyze for the customization of PCa treatment for individual patients.

Further study needs to be done to confirm and establish these achievements.

OTHER ACHIEVEMENTS

Successfully defended final Ph.D. dissertation on May 14, 2014, Department of Chemical Engineering, University of Rochester, Rochester New York.

(Please see the appendices)

REFERENCES

Aguirre-Ghiso, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. Nature reviews Cancer *7*, 834-846.

Barthel, S. R., Gavino, J. D., Descheny, L., and Dimitroff, C. J. (2007). Targeting selectins and selectin ligands in inflammation and cancer. Expert opinion on therapeutic targets *11*, 1473-1491.

Barthel, S. R., Gavino, J. D., Wiese, G. K., Jaynes, J. M., Siddiqui, J., and Dimitroff, C. J. (2008). Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin. Glycobiology *18*, 806-817.

Barthel, S. R., Hays, D. L., Yazawa, E. M., Opperman, M., Walley, K. C., Nimrichter, L., Burdick, M. M., Gillard, B. M., Moser, M. T., Pantel, K.*, et al.* (2013). Definition of molecular determinants of prostate cancer cell bone extravasation. Cancer research *73*, 942-952.

Bonnomet, A., Brysse, A., Tachsidis, A., Waltham, M., Thompson, E. W., Polette, M., and Gilles, C. (2010). Epithelial-to-mesenchymal transitions and circulating tumor cells. Journal of mammary gland biology and neoplasia *15*, 261-273.

Borsig, L., Vlodavsky, I., Ishai-Michaeli, R., Torri, G., and Vismara, E. (2011). Sulfated hexasaccharides attenuate metastasis by inhibition of P-selectin and heparanase. Neoplasia *13*, 445-452.

Dallas, M. R., Chen, S. H., Streppel, M. M., Sharma, S., Maitra, A., and Konstantopoulos, K. (2012). Sialofucosylated podocalyxin is a functional E- and L-selectin ligand expressed by metastatic pancreatic cancer cells. American journal of physiology Cell physiology *303*, C616-624.

Dimitroff, C. J., Descheny, L., Trujillo, N., Kim, R., Nguyen, V., Huang, W., Pienta, K. J., Kutok, J. L., and Rubin, M. A. (2005). Identification of leukocyte E-selectin ligands, P-selectin glycoprotein ligand-1 and E-selectin ligand-1, on human metastatic prostate tumor cells. Cancer research *65*, 5750-5760.

Dimitroff, C. J., Lechpammer, M., Long-Woodward, D., and Kutok, J. L. (2004). Rolling of human bone-metastatic prostate tumor cells on human bone marrow endothelium under shear flow is mediated by E-selectin. Cancer research *64*, 5261-5269.

Fukuda, M. N., Ohyama, C., Lowitz, K., Matsuo, O., Pasqualini, R., Ruoslahti, E., and Fukuda, M. (2000). A peptide mimic of E-selectin ligand inhibits sialyl Lewis X-dependent lung colonization of tumor cells. Cancer research *60*, 450-456.

Gajula, R. P., Chettiar, S. T., Williams, R. D., Thiyagarajan, S., Kato, Y., Aziz, K., Wang, R., Gandhi, N., Wild, A. T., Vesuna, F.*, et al.* (2013). The Twist Box Domain is Required for Twist1-induced Prostate Cancer Metastasis. Mol Cancer Res.

Gout, S., Morin, C., Houle, F., and Huot, J. (2006). Death receptor-3, a new E-Selectin counter-receptor that confers migration and survival advantages to colon carcinoma cells by triggering p38 and ERK MAPK activation. Cancer research *66*, 9117-9124.

Gout, S., Tremblay, P. L., and Huot, J. (2008). Selectins and selectin ligands in extravasation of cancer cells and organ selectivity of metastasis. Clinical & experimental metastasis *25*, 335-344.

Guo, G., Yao, W., Zhang, Q., and Bo, Y. (2013). Oleanolic acid suppresses migration and invasion of malignant glioma cells by inactivating MAPK/ERK signaling pathway. PloS one *8*, e72079.

He, H., Yang, X., Davidson, A. J., Wu, D., Marshall, F. F., Chung, L. W., Zhau, H. E., and Wang, R. (2010). Progressive epithelial to mesenchymal transitions in ARCaP E prostate cancer cells during xenograft tumor formation and metastasis. The Prostate *70*, 518-528.
Hidalgo, A., Peired, A. J., Wild, M. K., Vestweber, D., and Frenette, P. S. (2007). Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. Immunity *26*, 477-489.
Hsu, J. W., Yasmin-Karim, S., King, M. R., Wojciechowski, J. C., Mickelsen, D., Blair, M. L., Ting, H. J., Ma, W. L., and Lee, Y. F. (2011). Suppression of prostate cancer cell rolling and adhesion to endothelium by 1alpha,25-dihydroxyvitamin D3. The American journal of pathology *178*, 872-880.
Jacobs, P. P., and Sackstein, R. (2011). CD44 and HCELL: preventing hematogenous metastasis at step 1. FEBS letters *585*, 3148-3158.
Julien, S., Ivetic, A., Grigoriadis, A., QiZe, D., Burford, B., Sproviero, D., Picco, G., Gillett, C., Papp, S. L., Schaffer, L., *et al.* (2011). Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. Cancer research *71*, 7683-7693.
Lange, P. H., and Vessella, R. L. (1998). Mechanisms, hypotheses and questions regarding prostate cancer micrometastases to bone. Cancer metastasis reviews *17*, 331-336.
Laubli, H., and Borsig, L. (2010). Selectins promote tumor metastasis. Seminars in cancer biology *20*, 169-177.
Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nature reviews Immunology *7*, 678-689.
Li, J., Guillebon, A. D., Hsu, J. W., Barthel, S. R., Dimitroff, C. J., Lee, Y. F., and King, M. R. (2013). Human fucosyltransferase 6 enables prostate cancer metastasis to bone. Br J Cancer.
Mehta, P. B., Jenkins, B. L., McCarthy, L., Thilak, L., Robson, C. N., Neal, D. E., and Leung, H. Y. (2003). MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. Oncogene *22*, 1381-1389.
Mourelatos, Z., Gonatas, J. O., Nycum, L. M., Gonatas, N. K., and Biegel, J. A. (1995). Assignment of the GLG1 gene for MGF-160, a fibroblast growth factor and E-selectin binding membrane sialoglycoprotein of the Golgi apparatus, to chromosome 16q22-q23 by fluorescence in situ hybridization. Genomics *28*, 354-355.
Parsons, J. T., Horwitz, A. R., and Schwartz, M. A. (2010). Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nature reviews Molecular cell biology *11*, 633-643.
Sackstein, R. (2004). The bone marrow is akin to skin: HCELL and the biology of hematopoietic stem cell homing. The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research *9*, 215-223.
Sreeramkumar, V., Leiva, M., Stadtmann, A., Pitaval, C., Ortega-Rodriguez, I., Wild, M. K., Lee, B., Zarbock, A., and Hidalgo, A. (2013). Coordinated and unique functions of the E-selectin ligand ESL-1 during inflammatory and hematopoietic recruitment in mice. Blood.
Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B., and Vestweber, D. (1995). The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. Nature *373*, 615-620.
Taichman, R. S., Cooper, C., Keller, E. T., Pienta, K. J., Taichman, N. S., and McCauley, L. K. (2002). Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer research *62*, 1832-1837.

Tozeren, A., Kleinman, H. K., Grant, D. S., Morales, D., Mercurio, A. M., and Byers, S. W. (1995). E-selectin-mediated dynamic interactions of breast- and colon-cancer cells with endothelial-cell monolayers. International journal of cancer Journal international du cancer *60*, 426-431.
Turner, N., and Grose, R. (2010). Fibroblast growth factor signalling: from development to cancer. Nature reviews Cancer *10*, 116-129.
Zayzafoon, M., Abdulkadir, S. A., and McDonald, J. M. (2004). Notch signaling and ERK activation are important for the osteomimetic properties of prostate cancer bone metastatic cell lines. The Journal of biological chemistry *279*, 3662-3670.

APPENDICES

Please see 2 forwarded emails.

TRAINING OR FELLOWSHIP AWARDS

Opportunities for training and professional development the project has provided:

1. Allowed to complete the course work for the PhD program.
2. Provided the opportunity to one work with mentor Dr. YiFen Lee.
3. Allowed increased knowledge and skill by participating in the following conferences and seminars:

Poster presentation: American Urological Association (AUA), Annual Scientific Meeting, Orlando, Florida, May 16 – 21, 2014.

"E-selectin Ligand-1 Controls Circulating Prostate Cancer Cell Rolling/Adhesion and Metastasis".

Authors: **Sayeda Yasmin-Karim**, Michael R. King, Edward Messing and Yi-Fen Lee.

University of Rochester Graduate Research Showcase, University of Rochester, April 18, 2013.

"Capture and Analyzing of Prostate Cancer Stem cells using a Microcirculatory Channel".

Authors: **Sayeda Yasmin-Karim**, Huei-Ju Ting, Michael R. King, Edward Messing and Yi-Fen Lee.

American Urological Association (AUA), Annual Scientific Meeting, Atlanta, Georgia, May 19 – 23, 2012. "Capture and Analyzing of Prostate Cancer Stem cells using a Microcirculatory Channel".

Authors: **SayedaYasmin-Karim**, Huei-Ju Ting, Michael King, Edward Messing and Yi-Fen Lee.

- Participate in bi-weekly 'Journal –Club' study group.
- Participate in bi-weekly 'Lab-meeting' for working data presentation and discussion.
- Participate in weekly meeting with mentor Dr. YiFen Lee for research project discussion.

Ph.D Defense

Influence of Selectin Mediated Rolling/Adhesion Dynamics in Prostate Cancer Metastasis: a Micro-Channel Model

Sayeda Yasmin-Karim

Professor Yi-Fen Lee, Department of Urology, *Co-Advisor*

Professor David Wu, Department of Chemical Engineering, *Co-Advisor*

Abstract

Prostate cancer is the second most common cancer in men in America and metastasis is the cause of death in most patients. Circulating prostate cancer cells preferentially roll and adhere on bone marrow vascular endothelial cells where abundant E-selectin and stromal cell-derived factor 1 (SDF-1) are expressed, subsequently initiating a cascade of activation events that eventually lead to the development of metastases. To elucidate the importance of circulating prostate cancer cells' rolling and adhesion behaviors in the development of metastasis, we applied a dynamic cylindrical flow-based microchannel device that is coated with E-selectin and SDF-1, mimicking capillary endothelium, and allowing prostate cancer cells to roll and adhere under flow.

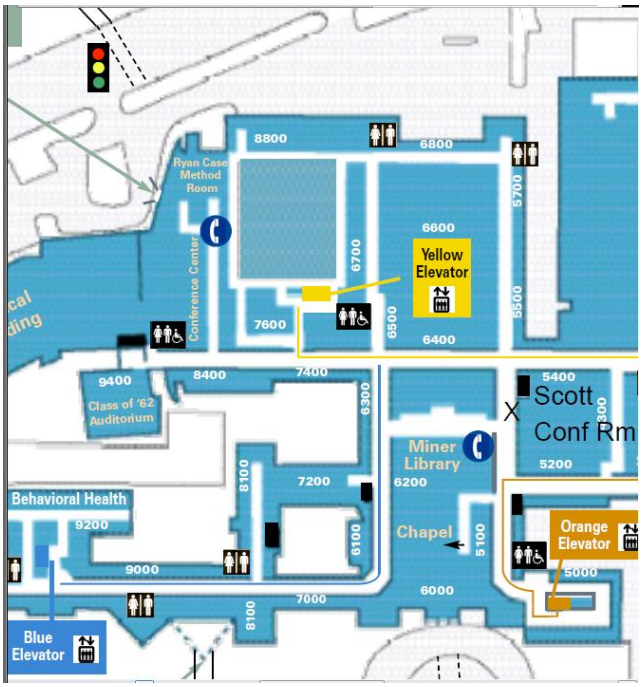
We reported that prostate cancer cells' rolling ability positively correlates with their aggressiveness. Using this device we were able to capture a small fraction of rolling prostate cancer cells and characterize their behavior and genetic contents. The rolling cells possess high adhesion ability and display aggressive cancer phenotype *in vitro* and *in vivo* compared to non-rolling/floating cells. Moreover, the expression level of genes associated with cancer stem cells and cancer metastasis was found to be elevated in rolling prostate cancer cells. Importantly, the expression of E-selectin ligand-1 (ESL-1) was overexpressed in rolling cells and knock down of ESL-1 significantly impaired prostate cancer cells' rolling capacity and cancer aggressive phenotypes. Detailed mechanism studies revealed that ESL-1 activates RAS, consequently inducing a signal cascade such as MAP kinase and its downstream targets to influence cell proliferation and migration. The elevated level of ESL-1 was also found in the metastatic prostate cancer patients, comparison to normal prostate and primary tumors, through the public microarray repositories from NCBI Gene Expression Omnibus.

In conclusion, ESL-1 and its induced oncogenic pathways represent new therapeutic targets that control circulating prostate cancer cells' rolling, adhesion and distant metastasis. This flow-based microchannel system represents a powerful way to capture a small population of the most aggressive group of circulating prostate cancer cells and provide a functional platform to develop a strategy to stop prostate cancer invasion by interrupting these circulating prostate cancer cells' rolling/adhesion ability.

Wednesday, May 14, 2014, 2:00 PM

W. W. Scott Conference Room (1-5336)

University of Rochester Medical Center



Conway, Patricia A

From: Yasmin-Karim, Sayeda
Sent: Monday, June 09, 2014 9:13 AM
To: Conway, Patricia A
Subject: FW: Celebration for Dr. Yasmin-Karim in Chen's Garden tomrrow noon.

From: Lee, YiFen
Sent: Wednesday, May 14, 2014 5:25 PM
To: Wu, Chia-Hao; Silvers, Christopher R; Yin, Peng-Nien; Hsu, Jong-Wei; Moses, Michael; Si, Shuhui; Conway, Patricia A; Steinorth, Deborah; Schoen, Sue
Cc: Yasmin-Karim, Sayeda
Subject: Celebration for Dr. Yasmin-Karim in Chen's Garden tomrrow noon.

Dear All,
Sayeda has passed her thesis defense today.
Yeah!! She did it!! We can now call her "Dr. Yasmin-Karim".

You are all invited to a celebration luncheon
When: tomorrow @12:15
Where: Chen Garden-- 1750 Monroe Ave, Rochester NY 14618

PS: To get a headcount, please let me know if you can be there!

Yi-Fen